

**INTRAVITAL MICROSCOPIC EVIDENCE THAT POLYLACTIDE-POLYGLYCOLIDE (PLGA)  
DELAYS NEO-OSTEOGENESIS AND NEO-ANGIOGENESIS IN HEALING BONE**

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**Abstract**

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**Key Words:** Bio-erodible implants, polylactide-polyglycolide, bone chamber window, intravital microscopy, bone defect, fracture healing, biocompatibility, osteogenesis, vascularity, angiogenesis, trabeculae.

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**Introduction**

A goal of orthopaedic surgery is the restoration of traumatized bone to a functioning status. Where trauma results in fractures, fixation devices are often required. Where radical resection of tumors or infected bone leaves a large defect, reconstruction requires grafting procedures using a range of implant materials, from vascularized autogenous bone to ceramics. If the implant is sufficiently tolerated to induce no worse than a sequestrum and the bone returns to an acceptable level of function, no further surgery is necessary. However, neither allogeneic nor autogeneic bone implants are ideal (Hollinger & Battistone, 1986) and many implants must be surgically removed. For reconstructions, revision may be necessitated by complications such as osteomyelitis. Fixators, in contrast, are prophylactically removed to preclude stress-shielding-induced resorption, among other complications.

Erodible implants have the theoretical potential to be replaced by host bone while they gradually shift mechanical stress to regenerating bone. They must, however, be osteoconductive, which is to say they must encourage bone to occupy the space created by their degradation quickly enough to maintain load transfer continuity and/or prevent soft tissue prolapse. Failure to do so in loaded bone would invite fracture. They also must be biocompatible, to the extent that they generate no significant foreign-body reaction. To be sure, "There is no such thing as an inert implant material or device. At best the host tissues will benignly tolerate an implant" (Bloch, 1984).

For almost 30 years, erodible polymers have been used, initially as "absorbable" sutures and more recently for orthopaedic fixation (Zhang et al., 1993) and reconstruction in place of metallic plates/screws, allogeneic/autogeneic bone or other erodible materials (Gilding & Reed, 1979; Visscher et al., 1985). The clinical rationale is that revision surgery will be unnecessary. Furthermore, erodible polymers would not be expected to elicit an antigenic response.

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Indeed, it is often assumed that the degrading polymer will induce no foreign-body reaction.

Aliphatic polyesters such as polylactide (PLA, in combination) and polyglycolide (PGA, alone or in combination) have been used widely as erodible sutures. Rokannen and his colleagues have applied various formulations of PLA and PGA in over 1600 orthopaedic fixations, taking advantage of their range of mechanical properties (Daniels et al., 1990). This range may be extended by forming copolymers of the two aliphatics in various molar ratios (e.g. 50:50 or 90:10). The resulting poly-L-lactide-glycolide (PLGA) can then undergo post-synthesis manipulation to further alter its degradation profile (Lewis, 1990). Consequently, based upon its chemical flexibility and successes in previous investigations (Hollinger, 1983; Hollinger & Battistone, 1986; Hollinger & Smitz, 1987), PLGA was the polymer of choice for the investigations reviewed here. It was expected that a 50:50 LA:GA composition would combine the mechanical strength of polyglycolide (Daniels et al., 1990) with the erosion stability of poly-L-lactide (Lewis, 1990).

Biocompatibility of these polymers in orthopaedic application has been called to question recently by reports of clinical non-specific aseptic foreign-body reactions (Böstman et al., 1991) and *in vitro* toxicity (Daniels et al., 1992). Accordingly, it was necessary to examine the interaction of PLGA implants and bone, using the BCI and intravital microscopy. Incorporation (the replacement of an implant by regenerating host bone) in the tissue was quantitated.

The project goal was to chronicle the appearance of specific tissues (trabeculae and vessels) in the BCI, thereby advancing understanding of the incorporation process.

#### **Intravital Microscopy for Observation of Incorporation**

Clinical studies do not allow one to follow the interaction of polymer and host bone under controlled conditions. *In vitro* studies do not recreate the dynamics of interstitial fluid exchanges and *in vivo* studies usually have been restricted to assessing harvested tissue. By implanting a BCI which acts as an *in situ* window to the incorporation focus, it is possible to follow the interaction of bone and polymer as cause and effect.

Optical bone chamber intravital microscopy provides the necessary window. The bone chamber implant (BCI) is a descendent of Sandison's (1924)

rabbit ear chamber. It was developed by Brånemark (Brånemark et al., 1964) and refined by Albrektsson and Albrektsson (1978) and McCuskey et al. (1971). It is a bicortical tibial screw which houses a chamber bounded on two sides by parallel, optically flat fused-silica elements, between which healing tissue may be observed as it converts from fibrovascular tissue to bone (Winet, 1989). The elements are 100µm apart. Histological studies using the BCI have been reviewed by Albrektsson (1987). The present review focuses on the quantitative chronic studies from the Bone Chamber Laboratory of Orthopaedic Hospital (OH-BCL) which have been conducted since 1986 and more recent work at USAIDR (U.S. Army Institute of Dental Research).

The components of the BCI are shown in Figure 1. They were glued together with blackened surgical grade silastic (RTV) and the unit sterilized with ethylene oxide at room temperature as described previously (Winet & Hollinger, 1993). Nineteen mature (>6 months old) male and female New Zealand White rabbits were utilized. They were all at least 4kg (Winet & Hollinger, 1993). Bicortical implantation followed irrigated burring at 1500 rpm and tapping. Both implantation and exposure surgeries, which occurred 20 days post-implantation (D20), were aseptic and accompanied by an antibiotic protocol. The three-week delay allowed for osseointegration of the implant. Pin-tract infection post-exposure was prevented by daily lavage.

Observations began three weeks (W3=D21) post-implantation and continued weekly until W8 or later. They were performed on a horizontal intravital microscope as shown in Figure 2. The microscopic field-of-view revealed a 2mm circle in the window. The image was termed a "slit-gap field-of-view" and its contents the "slit-gap tissue"; boundaries formed a slit and extended across the gap defect.

The field-of-view was recorded on photographic film and videotape. Apposition new bone ("neo-osteogenesis") was revealed by transmitted light illumination. Collagen orientation and mineralization were assessed with transmitted, polarized light and oxytetracycline epifluorescence. Perfused microvasculature, which the BCI is uniquely able to show, and neo-angiogenesis were detected with epifluorescence of intravenous-injected microspheres (1.75µm) and dextran-conjugated dyes.

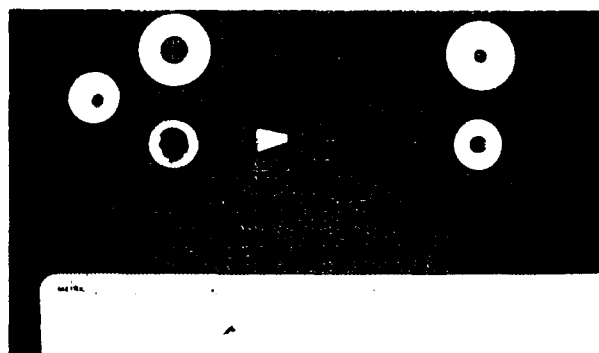
Measurements from the BCI were recorded over time and established a quantitative "normal" healing baseline for neo-osteogenesis and neo-angiogenesis. These observations determined the control data base

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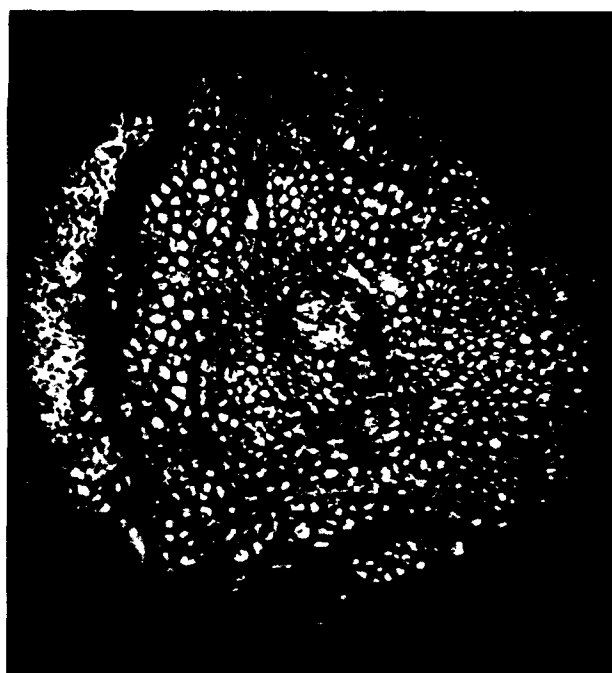
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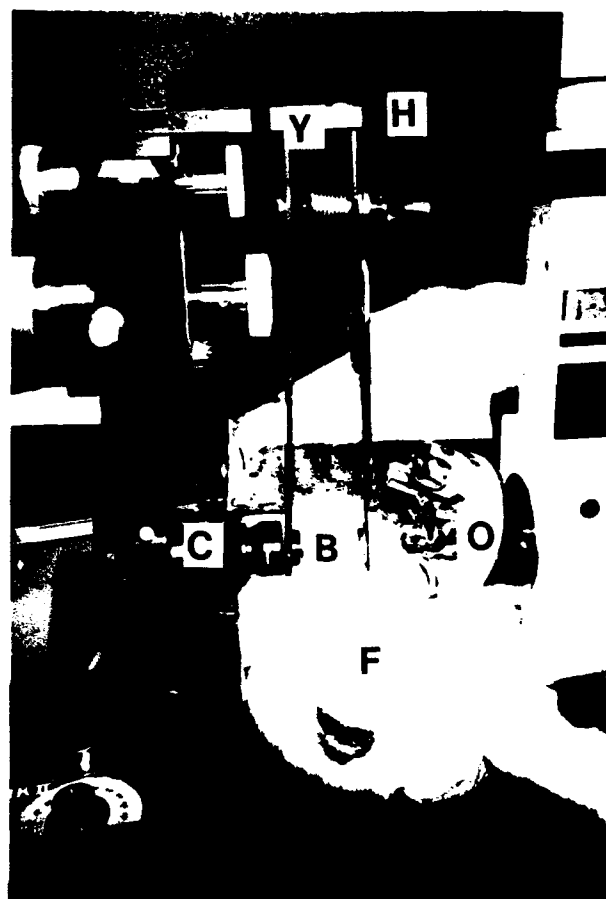
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**Figure 1.** Exploded view of bone chamber implant (BCI). At upper center is hollow titanium screw (30mm long), i.e. a shell. Below it are the quartz (fused-silica) window elements ground to optical flatness at each end. The quartz rod is glued into the tail and the cone into the head of the BCI. At their interface is a 100µm slit. The circular objects at the left and right are threaded buttons which screw on to the head or tail to keep skin reflected during permanent exposure for chronic viewing.



**Figure 3.** Slit-gap tissue at W90 (90 weeks following implantation). At this time there was no evidence of PLGA. Bone appears to be the dominant tissue. Cortical lamellar bone appears to be mixed with fat cells which may have migrated from the medullary canal as they were not seen during incorporation. Circle of image is 2mm in diameter.



**Figure 2.** Intravital microscope observation through the BCI. B= part of leg carrying BCI; C= microscope condenser for transmitted light; F= rabbit's foot; H= location of rabbit's head. O= microscope objective turret (Objective in same optical axis as BCI is about 1/2 the distance between 'B' and 'O'); Y= top of yoke which aligns and holds BCI in same optical axis as objective.

(Winet et al., 1990ab). Bone apposition was expressed as change in per cent of slit-gap field occupied by apposing trabeculae ( $\Delta\%B$ ) with time. Angiogenesis was expressed as vessel length per unit volume of matrix bone and/or fibrovascular tissue ( $L/V$ ) as a function of time.

There was concern that rigidity of the titanium shell of the BCI would stress-shield the slit-gap tissue, leading to resorption-related deviations from the normal healing process. Two lines of evidence have significantly reduced the validity of this concern. First, electrokinetic, not piezoelectric forces determine mineralization in bone (Otter et al, 1990). Consequently, fluid flow, which for bone blood vessels is determined more by mechanics outside (muscle

contraction, etc.) than inside the bone, has more impact on events in the BCI than do local bone stresses. Second, most chambers examined after over a year of implantation contain lamellar bone; an example of which is presented in Figure 3. If stress shielding were significant, bone would be either totally resorbed or would not be able to regenerate beyond primary osteons (i.e. woven bone).

### The BCI as a Tool for Studying PLGA Incorporation

Traditional studies of PLGA incorporation have been histological. Few have applied quantitative methods to study bone defects filled with PLGA plugs and harvested at various time intervals to assess the changes in cell and tissue populations (Hollinger, 1983). Bone apposition was quantitatively and vascularity qualitatively evaluated in these studies. Results indicated a PLGA-generated enhancement of bone healing (Hollinger, 1983).

Nevertheless, it was hypothesized for the BCI study that  $\Delta\%B/t$  would not be altered by PLGA erosion. It was reasoned that in the Hollinger studies, healing rat bone tissue was able to use the PLGA plug as a scaffolding. Moreover, the intimate contact between bone and plug shortened the distance invading vessels had to traverse with their acid-buffering plasma. In the BCI there was a space of about 1mm between the perimeter of the bur-hole and the PLGA—the total defect diameter was 4mm—that would probably serve as a reservoir for the accumulation of lactic and glycolic acid until blood vessels could cross into the slit-gap field-of-view. The form of 40kDa PLGA used was a 100 $\mu$ m diameter thread, a poor scaffolding candidate in a 2mm-wide tissue, even when penetrated by advancing vessels. The interaction of PLGA with tissue was postulated, consequently, to be more chemical than mechanical. Thus, it was postulated that the acidic monomers of PLGA degradation could accumulate in the reservoir sufficiently to inhibit neo-osteogenesis from the cortex. At this point the report by Vasenius et al. (Vasenius et al, 1992), showing that except for a point well after incorporation blood pH changed insignificantly around polylactide intramedullary rods, had not appeared.

How would polymer erosion effect the micro-vasculature? Lactic acid is a known stimulator of angiogenin release from macrophages (Jensen et al., 1986). Could PLGA degradation stimulate angiogenesis? Pyruvic acid, product of glycolic acid metabolism has no effect on angiogenesis. Thus, copolymer degradation would be expected to stimulate angiogenesis. Neo-osteogenesis is associated with high vas-

cularity (Heppenstall, 1980). Accordingly, PLGA erosion would have a positive effect on neo-osteogenesis via the vascular avenue. There appear to be no studies of the direct effect of PLGA bio-absorption on cytokine production.

The two conflicting predictions suggested a balance between agents either that favor or inhibit neo-osteogenesis. Consequently, it was hypothesized that their effects would offset and neo-osteogenesis into a BCI would occur at the same rate in controls (i.e., unloaded BCIs) as in experimentals (i.e., BCIs loaded with PLGA); which is, in effect the null hypothesis).

### The Control Model

In order to define a response to a foreign body, one must have a standard for comparison that excludes the foreign body, i.e., a control wound healing model for bone. Central to the establishment of such a model—particularly if it is to be quantitative—is the question of which physiological (or pathophysiological) parameters give the most insight to healing mechanisms. This question was answered on a practical level.

It was impractical to measure all physiological parameters in a control model, with the hope that one of them would be the key variable determining success of implant incorporation. Neo-osteogenesis is an obvious choice when bone is the implant site.

As in other forms of regenerative wound healing, however, success of osteogenesis is determined by effective deployment of cells which prepare the way for normal osteoblast function. Among these are macrophages (or their other existences as monocytes or histiocytes), fibroblasts and endothelial cells which regulate each other (and themselves) with a variety of cytokines (D'Amore, 1991). The BCI does not permit sufficient optical resolution to track individual cells (except some in the vessels) or their secretions without linking cell and/or secretion to fluorescent molecules (This aspect of bone intravital microscopy is in its infancy and is a fertile ground for future development of the model.). Observations thus far have had to be limited to the tissue level, however, and it is this consequence of wound healing cell action that has characterized BCI reports.

In the control BCI, macrophages essentially have completed their contribution to wound healing by W3 (the third week post-implantation). The tissue in the field-of-view at this time is fibrovascular (Albrektsson, 1987). Polarized microscopy has revealed birefringence patterns characteristic of oriented collagen fibers (Winet et al., 1990a), undoubtedly laid down by

# PLGA Effect on Regeneration in Bone Chamber

TABLE 1. Neo-Osteogenic Parameters

PARAMETER	CONTROL	EXPERIMENTAL
First detected	Between W3 and W4	Between W4 and W5
Maximum ACCUMULATION	W8 or W9 (in Bone only)	Between W10 and W12 (B)
OCCURRENCE of Maximum rate	Between W4 and W5	Between W7 and W8
VALUE of Maximum rate	$\Delta\%B = 3.6\%$ per day	$\Delta\%B = 4.3\%$ per day
OCCURRENCE of Minimum rate	When slit-gap is filled	When slit-gap is filled
VALUE of Minimum rate	Apparently Zero when slit-gap is filled	Apparently Zero when slit-gap is filled

the fibroblasts and providing the scaffolding for endothelial cells. Many of the vessels formed by the endothelial cells were sufficiently interconnected to sustain a pressure gradient as evidenced by flowing blood, made visible by intravenously-injected 70kDa fluorescein isothiocyanate dextran.

Videotapes of these observations were analyzed using a digital image processing system. Projected bone area measures were converted to volume values by assuming a thickness equal to the slit (Each slit was measured following chamber construction.). By a similar procedure, vessel length was obtained from fluorescent images and expressed as length per unit volume (L/V) of encompassing tissue.

"Neo-osteogenesis" and "neo-angiogenesis" observations from the control and experimental BCLs are summarized in Tables 1 and 2. The prefix "neo" is used as the regenerating tissue is entering a "new" space, the bone chamber, while at the same time refilling an old space (the bone defect). "Angiogenesis" is used in place of "vascularization" because the mechanism of healing, as opposed to a mere description of structure, is the main interest. The control data, summarized in the Tables have been reported (Winet et al., 1990ab).

Trabeculae were usually detectable between W4 and W5. The slope of neo-osteogenesis plot, expressed as percent of slit-gap filled per day, reached a maximum of 3.6% at this time. Between W8 and W9 bone was the only tissue apparent in the BCL, and it was assumed, based upon control data (Winet et al.,

TABLE 2. Neo-Angiogenic Parameters

PARAMETER	CONTROL	EXPERIMENTAL
First detected	Prior to W3	Prior to W3
Maximum ACCUMULATION	1. In fibrous tissue, no maximum L/V 2. In osseous tissue, at W8	Between W10 and W11
OCCURRENCE of Maximum rate	Between W7 and W8	Between W6 and W9
VALUE of Maximum rate	$L/V = 3.80 \times 10^6 \mu\text{m}/\text{cc}$ per day	$L/V = 5.12 \times 10^5 \mu\text{m}/\text{cc}$ per day
OCCURRENCE of Minimum rate	Between W3 and W4	Between W6 and W9
VALUE of Minimum rate	$L/V$ decreases $2.42 \times 10^6 \mu\text{m}/\text{cc}$ per day	$L/V$ decreases $2.73 \times 10^5 \mu\text{m}/\text{cc}$ per day

1990a), to be the only tissue present.

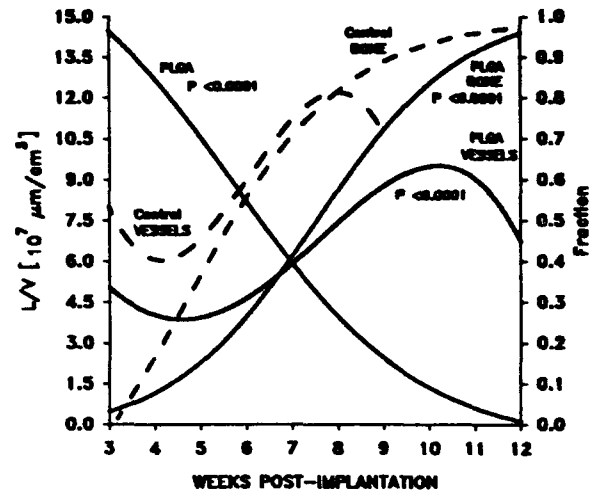
The vessels observed were not the same from week to week. It was unusual to be able to identify a vessel at one week that was clearly the same vessel observed the week before. Thus, the neo-angiogenesis described herein is not a simple lengthening of vessels. It may well be the net effect of vessel sprouting and resorption. The term 'neo-angiogenesis' in this model at least, must be understood within the limits of these considerations, and the fact that only perfused vessels were measured. Neo-angiogenesis followed a pattern not previously reported for bone. Post-traumatic hyperemia, characteristic of the inflammatory phase of wound healing was evidently accompanied by high vascularization and perfusion in the slit-gap fibrovascular tissue. As these vessels were resorbed, L/V decreased, an effect still in progress at W3. Accordingly, a descending curve of L/V vs time was typical between W3 and W4. Maximum neo-angiogenesis occurred between W7 and W8. A comparison of invading trabeculae with their host fibrovascular matrix showed that this L/V increase was contributed by the ossified component. In this model, at least, apposing bone brings blood to the healing tissue. When perfused vasculature was not extensive, bone apposition was high. As apposition leveled off to a zero rate—with remodeling replacing modeling—neo-angiogenesis decreased.



**Figure 4.** Neo-osteogenesis, neo-angiogenesis and PLGA erosion in a bone chamber. First panel shows polymer pre-implantation. Other panels are vertical pairs of brightfield and fluorescent images. Times (W3, etc.) are in weeks post-implantation. Polymer (P), trabeculae (B) and fibrovascular tissue (F) are labeled in the first brightfield row. Perfused vessels (PV) are parallel with the bone long axis. W10 is represented with two fluorescent photographs with the brightfield image replaced by oxytetracycline deposition at mineralization sites (arrow) and regions from which it has not yet diffused (photograph taken less than 4 days after injection).

### The Experimental Model

Neo-osteogenic response to PLGA in the BCI has been reported (Winet & Hollinger, 1993). One of the first discoveries was an apparent rapid degradation in a small but significant number of polymer strands *in vivo*. This result was attributed to the existence of highly amorphous sections of the copolymer and serves as an indicator of variability in the syntheses of these polyesters. No healing occurred in these bone chambers.



**Figure 5.** Summary of measurements of neo-osteogenesis and neo-angiogenesis in control and experimental subjects, and PLGA erosion in the latter. Vascularity is defined as perfused vessel length per unit tissue volume. Ossification is fraction of slit-gap field-of-view filled with trabeculum. Error bars are omitted for clarity, but may be found in original reports. The control bone regression curve has been continued well beyond W9 (the final observation point if beyond W8) for symmetry. Note that neo-osteogenesis is delayed and neo-angiogenesis is both delayed and inhibited around the eroding PLGA.

Results from the more typical specimens are depicted in Figure 4. Data from these observations are summarized in Tables 1 and 2 and Figure 5. Trabeculae usually appeared at W5, occasionally being delayed to W6 or W7. Osseous filling of the slit-gap was rarely achieved before W10 and the maximum neo-osteogenic rate occurred between W7 and W8. The delay in comparison with the controls was statistically significant between W5 and W9. After this delay, however, osteogenesis, as indicated by the curve slope, proceeded at the same rate as the controls. An apparent 'recovery' of osteogenesis after W5 produced apposition rates as high as 4.3%, a value 20% higher than the fastest control rate. Such accelerations were not, however, sufficiently characteristic to generate a steeper regression curve (Figure 5).

There was no evidence of incompatibility between trabeculae and polymer. It appeared that the regenerating bone interfaced directly with the polymer (Figure 4). Confirmation of this observation at the cellular level awaits further histological study.

Neo-angiogenesis analysis is still in preliminary stages but it was apparent that L/V vs time was



delayed in the presence of PLGA. Maximum perfused vascularity occurred between W10 and W11, three weeks after the control peak. Neo-angiogenesis was depressed, with the minimum L/V value almost halved (Figure 5). Nevertheless, the pattern of neo-angiogenesis persisted, including a projected maximum L/V prior to W3. Probable linkage of the vascular and bone results is supported by recent observations of Brighton et al. (1991), that osteogenesis is enhanced by low and collagen synthesis by high  $PO_2$ . At this time separate evaluation of trabecular and fibrovascular L/V has not been performed. It appears, however, that while the delay in neo-angiogenesis can be explained as an effect of trabecular apposition delay, the cause of its inhibition is more elusive. Further analysis will be necessary to determine if vascularity in the trabeculae has significantly changed in the presence of eroding PLGA.

No correlation could be found between the disappearance of PLGA and rate of neo-osteogenesis or neo-angiogenesis. The plots in Figure 5 suggest a negative correlation between bone apposition and polymer erosion. If the study had been performed using acute observations, a statistically significant linkage would have calculated. The advantage of a chronic model like the BCI, is that each rabbit serves as its own reference. Resulting correlations were, accordingly, animal-specific and, consequently, a more accurate reflection of cause-and-effect.

Biocompatibility appeared to be supported by neo-angiogenesis patterns. There was often evidence of vessel penetration of polymer fractures (Figure 4). Nevertheless, neo-osteogenic and neo-angiogenic delay during PLGA erosion was significant. There is an apparent paradox between this circumstantial relationship and the lack of correlation between the disappearance of PLGA and rates of the two regeneration processes cited above. The paradox may be resolved by postulating that one of the two apparently time-dependent processes is not truly time-dependent. Since tissue regeneration is demonstrably time-dependent (Winet et al., 1990ab), the candidate must be the PLGA. A scenario in which the visible erosion of polymer is not representative of the actual loss of mass is not difficult to fashion for bulk eroders such as PLGA. Visible erosion readings are based on changes in the polymer's profile. Bulk eroders degrade internally before their outer boundary, which apparently acts like a semi-permeable membrane (Vert et al., 1992), shows any sign of absorption. Accordingly, monomer efflux through the 'membrane' which results in polymer weight loss and may influence tissue regeneration, is not reflected in visible polymer surface changes. This shortcoming of the BCI model may be surmounted by incorporating into polymer

structure a covalently bound fluorescent dye which would be released only by depolymerization.

The retardation of bone and vessel regeneration did not appear to be caused by the mere geometry of a foreign object in the slit-gap field, because there was sufficient room for both vessels and bone before either contacted the polymer surface. Moreover, a persistent effect would have diminished, rather than delayed, the two regenerations. The fact that neo-osteogenic rates 'recovered' following their delay and neo-angiogenic plots retained their two-peaked pattern, supports the conclusion that normal homeostatic wound healing mechanisms were intact in the slit-gap tissue. Although the PLGA strands were too small to pressure the surrounding tissue when expanding, they could have generated crystalline "wear" particles during erosion. Accordingly, there remain mechanical effects which cannot be ruled out.

Of the possible chemical effects, acidosis is not supported by recent observations on PLA *in vivo* (Vasenius et al., 1992) and PLGA *in vitro* (Li et al., 1990). However, pH in the PLA study was measured in nearby blood vessels rather than near the eroding surface. Thus, there is as yet no accounting for relatively unbuffered, truly local effects. In the present study the expected stimulation by lactic acid monomers of neo-angiogenesis did not materialize. A reduction of macrophages by W3 may have been the reason. In any case, bio-incompatibility—much less toxicity—did not appear to be a significant factor in these studies. This concurs with other rabbit studies (Vasenius et al, 1992). Specific chemical interactions yet to be investigated include the role of blood-borne agents and their rate of delivery on the erosion process. Accordingly, analysis of permeability and blood supply in the BCI-PLGA model are currently in progress to obtain insight into the transport aspects of the incorporation of this copolymer.

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In conducting this research, the investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and

use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication #86-23, Revised 1985).

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## PLGA Effect on Regeneration in Bone Chamber

### Discussion With Reviewers

**T.A. Einhorn:** Is there any evidence that PLGA, or any of the other polymers currently used in the manufacture of resorbable sutures or implants, have a direct inhibitory effect on the synthesis of any cytokines and growth factors produced by osteoblastic, pre-osteoblastic, chondroblastic and inflammatory cells?

**Authors:** The short answer is that there do not appear to be any studies addressing the direct effect question in terms of synthesis. The long answer is that biocompatibility and osteoconduction have been the foci of both *in vitro* and *in vivo* studies. *In vitro* studies are exemplified by Laurencin's group (e.g. Elgendy et al., (1993) measured alkaline phosphatase activity in MC3T3-E1 osteoblast-like cells grown in culture on aliphatic polyesters and polyanhydrides.) An *in vivo* counterpart would have to relate the release of monomers to a change in production of a given cytokine. General effects of pH, as cited in the present report, have been studied for a number of cytokines. But, as shown by Jensen et al. (1986) who pinpointed lactic acid as the stimulus for macrophage angiogenin secretion, the specific molecule causing the observed effect must be known to interpret the data.

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